Coil Planet Centrifugal and Capillary Tube Centrifugal Analysis of Factors Regulating Erythrocyte Osmotic Fragility and Deformability

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Abstract Hydrated and dehydrated red cell samples were prepared from normal human red cells using the antibiotic nystatin. Furthermore, a series of red cell samples exposed to elevated temperature (20–50°C, 10 min) were prepared. The osmotic fragility and deformability of these red cells were then measured, using the coil planet centrifuge system and the capillary tube centrifugal technique, respectively. The osmotic fragility of nystatin-treated red cells decreased and the deformability increased as dehydration of red cells progressed and alternatively, hydrated cells showed increased osmotic fragility and reduced deformability. Red cells exposed to elevated temperatures up to 49°C for 10 min had no changes in mean corpuscular volume or in red cell shape. Above 47°C, however, spectrin extractability progressively decreased and osmotic fragility and deformability decreased. Results suggest that the osmotic fragility and deformability of red cells are interrelated, and are controlled by the geometry of the cell, including the ratio of cell surface area to cell volume and the viscoelastic properties of the membrane.

Key Words: red cell, deformability, osmotic fragility.

The measurements of erythrocyte osmotic fragility and deformability are useful in screening for hemolytic anemias and are applied to studies of membrane properties. Parpart's method for the osmotic fragility test (PARPART *et al.*, 1947) is a simplified means of estimating the surface area to volume ratio of erythrocytes, but it is a very time consuming procedure as compared with the coil planet centrifuge method (ITO *et al.*, 1966). The deformability of erythrocytes has been shown to be an important factor in reducing the bulk viscosity of blood flowing through vessels and it plays a crucial role in the rheology of blood circulation in the body. BESSIS and MOHANDAS (1975) devised the ektacytometer (a laser diffractometric technique) for the measurement of cellular deformability and ALLARD *et al.* (1978) reported using the ektacytometer so that erythrocyte deformation is determined by three major factors: 1) the viscoelastic properties

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of the membrane, 2) the surface area to volume ratio and, 3) the intracellular viscosity. In the present study we prepared hydrated and dehydrated red cells possessing normal membrane permeabilities and heated red cells possessing normal cell shape and mean corpuscular volume, thus designing investigation of the regulating factors of erythrocyte osmotic fragility, deformability, and their relationship using the coil planet centrifuge system and capillary tube centrifugal technique.

MATERIALS AND METHODS

Preparation of hydrated and dehydrated red cells. Healthy human bloods were drawn into heparinized syringes and the water content of normal red cells was changed by treating with the permeabilizing antibiotic nystatin. To alter the intracellular ion concentration, media containing 27 mM sucrose, 1.2 mM NaCl, and Z mM KCl (Z=75, 100, 125, 150, 175, 200, and 250) were used (CASS and DALMARK, 1973). Incubation with nystatin 50 μ l/ml (Sigma Chemical Company, U.S.A.) at 4°C for 1 hr permitted equilibration of potassium across the membrane. To remove the nystatin the cells were washed three times in nystatin-free medium having the same ion concentration finally reached within the cells and resuspended in isotonic phosphate-buffered saline (0.12 M NaCl, 0.020 M Na_2HPO_4 , 0.005 M KH_2PO_4 , 290 mOsm, pH 7.4). Hydration or dehydration of red cells occurred depending on the intracellular potassium concentration and no hemolysis was found during nystatin treatment.

Density distribution of hydrated and dehydrated red cells. Hydrated and dehydrated cells were fractionated on discontinuous gradients of polyvinylpyrrolidone-coated colloidal silica matrix (Percoll; Pharmacia Fine Chemicals AB, Uppsala, Sweden). A stock solution of isotonic Percoll was prepared by the addition of 1 part 10 $\times$ Rabinowitz's calcium- and magnesium-free phosphate-buffered saline (pH 7.4) to 9 parts Percoll, and this was diluted in Rabinowitz's buffered saline (RABINOWITZ, 1965) to prepare solutions with different density values. Gradients consisted of 10 layers ranging from 1.080 to 1.116 g/ml. In a typical separation, each 0.5 ml volume of Percoll density media was carefully overlaid in the tube with Pasteur pipettes at 4°C and 0.5 ml of sample was layered onto the discontinuous gradients; the tube was then centrifuged at 800 g for 20 min at 4°C.

Heating and spectrin extractability. Red cell suspension was added to the prewarmed phosphate-buffered saline at the specified temperature. The hematocrit was 50%. After incubation for 10 min the cell suspension was cooled in ice. For spectrin extraction red cell ghosts were prepared by hypotonic lysis (DODGE *et al.*, 1963) and isolated ghost membranes were suspended in 40 volumes of 0.1 mM EDTA, pH 8.0 (FAIRBANKS *et al.*, 1971) and incubated at 37°C for 20 min. The extracted pellets were centrifuged for 30 min at 10⁴ g. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed

as previously described (FAIRBANKS *et al.*, 1971) on membrane proteins using 5% polyacrylamide gels. Spectrin extractability was evaluated by scanning the SDS-PAGE gels of extract pellets and determining the ratio of spectrin: Band 3 from the densitogram.

Dynamic measurement of osmotic fragility of red cells. The osmotic fragility of red cells was measured at 37°C by the coil planet centrifuge as previously described (ITO *et al.*, 1966; NAGASAWA *et al.*, 1976). Ten μ l of red cell suspension in isotonic phosphate-buffered saline (hematocrit, 50%) were applied to the coiled tube containing saline with an osmotic gradient. The hemolytic patterns were recorded with a scanning photodensitometer.

Centrifugal measurement of red cell deformability. Red cell deformability was measured by a modified capillary tube centrifugal method (NAGASAWA *et al.*, 1980) of CORRY and MEISELMAN (1978). To observe the deformation of red cells, 5 μ l of whole blood were suspended in 5 ml of Eagle-MEM (Minimum Essential Medium Eagle with Earle's balanced salt solution) and 50 μ l of this suspension were placed in the top portion (1.5 cm) of the siliconized capillary tube (8 cm length, 2 mm inner diameter). The middle portion (5 cm) of the tube contained isotonic phosphate-buffered saline (290 mOsm, pH 7.4). In the bottom portion (1.5 cm) phosphate-buffered 1% glutaraldehyde solution (290–295 mOsm, pH 7.4) was present to fix the deformed cells. The capillary tube was quickly spun at 12,000 rpm for 1 min at 37°C using the hematocrit centrifuge (Marusan centrifuge Type B-L, Sakuma Co., Ltd., Tokyo) and the red cells were subjected to the centrifugal fields which induced cell deformation. The resulting deformation was evaluated by measuring the elongation of the red cells by a phase contrast microscope.

Measurement of mean corpuscular volume (MCV) of red cells. MCV was calculated by dividing the hematocrit value obtained using the spun hematocrit method (12,000 rpm, 5 min) by red cell count.

RESULTS

Density distribution of hydrated and dehydrated cells

Morphological examination of the dehydrated cells showed a slight flattening of disc and the hydrated cells were progressing from thicker discs to slightly cupped cells. The distribution of nystatin-treated red cells with abnormal water content on discontinuous Percoll gradients is shown in Fig. 1. The water content of normal red cells was varied by changing the intracellular potassium in the presence of the permeabilizing antibiotic nystatin and resuspending them in isotonic phosphate-buffered saline. The dehydrated and hydrated red cells were layered on the higher and lower densities, respectively, shown from left to right in the photograph.

Fig. 1. Density distributions of hydrated and dehydrated red cells by discontinuous Percoll density gradients. Red cells were treated with nystatin in (a) 75, (b) 100, (c) 125, (d) 150, (e) 175, (f) 200, and (g) 250 mM KCl solutions.

Osmotic fragility and deformability of hydrated and dehydrated cells

The dynamic measurement of osmotic fragility of hydrated and dehydrated red cells was performed using the coil planet centrifuge (Fig. 2). The dehydrated cells (Fig. 2a–c) show osmotic resistance and the progressively hydrated cells (Fig. 2e–g) increase osmotic fragility with a progressive shift of the hemolytic curve to higher sodium chloride concentration. The deformability of dehydrated and hydrated cells were measured by the capillary tube centrifugal technique, and the deformed shapes of dehydrated and hydrated cells are shown in Fig. 2 top to bottom corresponding to their osmotic fragility curves. The lengths of elongated cells in the longitudinal axis *versus* the MCV values are shown in Fig. 3. From the results shown in Figs. 2 and 3, cellular dehydration produced increased osmotic resistance and deformability and alternatively, hydration induced the cells to be more fragile and less deformable.

Heat effects on osmotic fragility, deformability, and membrane spectrin

The effects of elevated temperature on osmotic fragility of erythrocytes as measured by the coil planet centrifuge are shown in Fig. 4. Up to 40°C, erythrocytes heated for 10 min produced no changes in osmotic fragility. From 47 to 49°C red cells showed a temperature-dependent decrease in osmotic fragility and at 50°C, however, they suddenly became fragile. The mean corpuscular volume values of heated red cells were measured by the spun hematocrit method.

Fig. 2. Osmotic fragility curves of nystatin-treated red cells in (a) 75, (b) 100, (c) 125, (d) 150, (e) 175, (f) 200, and (g) 250 mM KCl solutions. Microscopic photos of typical cell elongation in centrifugal fields (12,000 rpm) are shown corresponding to their osmotic fragility curves.

Up to 49°C no changes were observed in MCV and all the cells were discocytes on microscopic observation. At 50°C red cells began to be sphere and fragment.

Figure 5A shows the effects of heating for 10 min at various temperatures on deformability of erythrocytes as measured by the capillary tube centrifugal technique. Above 46°C, red cells showed a progressive temperature-dependent decrease in deformability. Spectrin is rapidly released from the isolated erythrocyte membranes in very low ionic strength media (FAIRBANKS *et al.*, 1971). Figure 5B shows that about 80% of the material in the spectrin position is released from membranes of heated erythrocytes up to 46°C, while heating about 47°C causes a progressive decrease in spectrin extractability with increasing temperature and spectrin becomes unextractable at 49°C. These results reveal that heating

Fig. 3. Deformability of hydrated and dehydrated red cells. Deformability was evaluated as the length of cellular elongation in the centrifugal fields.

Fig. 4. Osmotic fragility curves of heated red cells at the indicated temperatures measured by the coil planet centrifuge system.

at 47 to 49°C for 10 min produces changes in osmotic fragility, deformability, and membrane organization, but not in morphology.

Fig. 5. Deformability and spectrin extractability of heated red cells. (A) Cellular elongation measured by the capillary tube centrifugal method. (B) Extent of spectrin extraction from ghost membranes prepared from erythrocytes exposed for 10 min at the indicated temperatures.

DISCUSSION

Changes in the osmotic fragility characteristics of red cells have been used to diagnose and differentiate various hemolytic disorders (BEUTLER, 1977; MIALE, 1972) and to investigate physiological membrane properties (CHAN *et al.*, 1975; DANON, 1963; DAVIES *et al.*, 1968; WESSELS and VEERKAMP, 1973, WESSELS *et al.*, 1973; ZADE-OPPEN, 1968). Parpart's method (PARPART *et al.*, 1947) and Danon's method (DANON, 1963) used to measure the osmotic fragility of red cells are not suitable for analytical study because of the non-linearized fragiligraph. To improve on this disadvantage, LEON *et al.* (1970) reported fragiligraph linearization using a computer program to evaluate the toxic or hemolytic actions of drugs and chemicals as well as exotic space-cabin atmospheres. In our study we used the coil planet centrifuge system to measure the osmotic fragility of red cells and obtained a linearized fragiligraph. BEUTLER (1977) has described that the osmotic fragility depends on the surface/volume ratio of erythrocytes. To clarify the regulating factors of osmotic fragility of red cells, we prepared hydrated and dehydrated red cells with a normal ion permeability using the antibiotic nystatin, and heated red cells possessing normal shape, normal MCV and membrane spectrin abnormality. From our experimental results using these red cells, the coil planet centrifuge detected alterations in the surface area to volume ratio and in the viscoelastic properties of the membrane.

Red cell deformability has been measured by the micropipette technique (EVANS and LACELLE, 1975), filtration (CHIEN, 1977), resistive pulse spectroscopy (MEL and YEE, 1975), automated ektacytometer (BESSIS *et al.*, 1980), centrifugal

elongation (CORRY and MEISELMAN, 1978; NAGASAWA *et al.*, 1980), and so forth. According to the ektacytometric study (ALLARD *et al.*, 1978), red cell deformability was regulated by three factors: internal viscosity, surface area to volume ratio, and viscoelastic modules of the membrane. In the present study regulating factors of deformability were evaluated by the capillary tube centrifugal technique using nystatin-treated red cells and heated red cells. Centrifugal forces deformed the red cells to a handbag-like form. In this shape, the flattened end portion of the cell becomes free of cell contents (CORRY and MEISELMAN, 1978). The degree of displacement of the membrane depends on the membrane deformability since this elongated flattened membrane portion is not affected by the internal viscosity of red cells. When dehydrated cells were studied, they showed increased cellular elongations in the centrifugal fields, depending on their increased surface area to volume ratio. When heated red cells were used, altered membrane properties caused changes in red cell deformation. Experimental results using the capillary tube centrifugal technique indicated that the red cell deformability was predominantly regulated by the surface area to volume ratio of the red cells and by the viscoelasticity of the membrane.

In conclusion, it was revealed by the coil planet centrifuge and the capillary tube centrifugal technique that the osmotic fragility and deformability of red cells were controlled by the relationship of the surface area to volume of the cell and the intrinsic rigidity of the red cell.

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